KOREAN PATENT APPLICATION
UNDER SERIAL NO. 10-2003-0076967

I, THE UNDERSIGNED, HEREBY DECLARE :
THAT I AM CONVERSANT WITH BOTH THE KOREAN AND THE ENGLISH LANGUAGES : AND

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KOREAN PATENT APPLICATION UNDER

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IN THE NAME OF: KOREA INSTITUTE OF SCIENCE AND TECHNOLOGY, et al.

FOR: DDR2 PROTEIN WITH ACTIVATED KINASE ACTIVITY AND PREPARATION METHOD THEREOF

IN WITNESS WHEREOF, I SET MY HAND HERETO

THIS 17TH DAY OF JULY, 2008

BY

OH, YE JIN

Application NO. 10-2003-0076967

[Name of Document] Amendments

[Object to be amended]	Claim 1
[Way of amendment]	Cancel
[Object to be amended]	Claim 2
[Way of amendment]	Cancel
[Object to be amended]	Claim 3
[Way of amendment]	Cancel
[Object to be amended]	Claim 4
[Way of amendment]	Change
[Contents amended]	

[Claim 4] A method for preparing DDR2 protein having a tyrosine phosphorylation induced kinase active domain, the method comprising:

amplifying cDNA encoding an amino acid sequence covering the kinase active domain of the DDR2 protein, introducing the amplified cDNA into a virus, and generating a recombinant virus encoding the DDR2 protein;

amplifying cDNA encoding an amino acid sequence covering Src protein, introducing the amplified cDNA into a virus, and generating a recombinant virus encoding the Src protein;

simultaneously infecting the DDR2 protein encoding recombinant virus and Src protein encoding virus into a host cell, followed by co-expression, and expressing the DDR2 protein having the tyrosine phosphorylation induced due to the tyrosine kinase activity of the co-expressed Src protein; and

purifying the tyrosine phosphorylation induced DDR2 protein.

[Object to be amended] Claim 5

[Way of amendment] Change

[Contents amended]

[Claim 5] The method of claim 4, wherein in the generating of the DDR2 protein encoding recombinant virus, the purification of the expressed DDR2 protein is facilitated by:

binding glutathione-S-transferase gene for an affinity tagging to cDNA encoding an amino acid sequence covering the kinase active domain of the DDR2 protein to be introduced into a virus;

generating the recombinant virus encoding the DDR2 protein and the glutathione-S-transferase; and

expressing the DDR2 protein in the form of a fused protein with the glutathione-S-transferase.

[Object to be amended] Claim 7

.[Way of amendment] Change

[Contents amended]

[Claim 7] The method of claim 4 or 5, wherein the cDNA covering the tyrosine kinase active domain of the DDR2 protein includes a DAN fragment which encodes the amino acids from position 441 to position 855 among cDNA encoding human DDR2 protein.

[Translation]

ABSTRACT OF THE INVENTION

[Abstract]

Provided are a method for phosphorylating a tyrosine of a DDR2 kinase active domain of DDR2 protein using a Src tyrosine kinase activity, a DDR2 protein with a tyrosine kinase active domain having an increased activity due to the tyrosine phosphorylation by the method, and the use of the DDR2 protein as a target protein in developing a medical drug for treating the diseases caused by the tyrosine-phosphorylated DDR2 protein. The protein containing the DDR2 kinase active domain having the tyrosine phosphorylation induced due to the Src tyrosine kinase can be useful for discovering a medical drug in developing new drugs for treating the diseases caused by an excessive activity of the DDR2 tyrosine protein.

[Representative Drawing]

Figure 5

[SPECIFICATION]

[Title of the Invention]

DDR2 PROTEIN WITH ACTIVATED KINASE ACTIVITY AND PREPARATION METHOD THEREOF

[Brief description of the Drawings]

Figure 1 shows the result determined by SDS-PAGE of the purified fused protein (GST-DDR2KD) of glutathione-S-transferase (GST) and DDR2 kinase active domain (DDR2KD).

Figure 2 shows the result obtained by checking that Src protein is expressed in case of infecting the prepared baculovirus expressing Src gene into a host cell through a western blotting using a Src protein specific antibody.

Figure 3a shows the result obtained by checking the induction of tyrosine phosphorylation of the DDR2 kinase active domain due to Src, which shows the results obtained by solely expressing the GST fused DDR2 kinase active domain protein or co-expressing with Src in host cells at a ratio of 1:1, purifying each obtained GST fused DDR2 kinase active domain protein to be subjected to an electrophoresis in PAGE gel, and performing the western blotting using a phosphorylated tyrosine specific antibody, after and then purifying,

Line 1: Protein purified after the sole expression of GST-DDR2; and

Line 2: Protein purified after the co-expression with Src.

Figure 3b shows that tyrosine phosphorylation of the DDR2 kinase active domain is induced due to Src, while the tyrosine phosphorylation is not induced when using GST-DDR1, GST-Akt1 and GST-CDK4, which shows the

experimental results obtained through the same method of Fig. 3a with respect to GST-DDR2, GST-DDR1, GST-Akt1 and GST-CDK4.

Figure 4 shows the result obtained by checking the variations of DDR2 protein expression depending on the combination ratio of DDR2 encoding virus and Src encoding virus when co-infecting baculoviruses each encoding the DDR2 kinase active domain and the Src into host cells, which shows the result obtained by performing a coomassie staining after purifying the DDR2 protein, and checking the tyrosine phosphorylation of the DDR2 kinase active domain through the western blotting using the phosphorylated tyrosine specific antibody.

Figure 5 shows the kinase activity difference between the DDR2 kinase active domain modified by the tyrosine phosphorylation due to the co-expression of the Src and the DDR2 kinase and the DDR2 kinase active domain in which the tyrosine phosphorylation is not induced,

Line 1: Protein purified after the sole expression of GST-DDR2; and

Line 2: GST-DDR2 protein purified after the co-expression with Src.

Figure 6 shows the variations of Km and Vmax in measuring the tyrosine kinase activities depending on variations of ATP concentrations of the DDR2 kinase active domain modified by the tyrosine phosphorylation due to the Src and the non-phosphorylated DDR2 kinase active domain, by way of purifying DDR2 kinase active domain proteins respectively obtained after the sole expression of the DDR2 kinase active domain protein or co-expression with the Src in host cells.

[Detailed description of the invention]

[Object of the invention]

[Field of the invention and background art]

The present invention relates to a method for phosphorylating a tyrosine of DDR2 kinase active domain of DDR2 protein using Src tyrosine kinase activity, a DDR2 protein containing a tyrosine kinase active domain having an increased activity due to the tyrosine phosphorylation by the method, and the user of the DDR2 protein in developing medical drugs for treating diseases caused by the DDR2 activity.

One of the ways that cells recognize an external stimulus is to recognize through receptor tyrosine kinase family present on cell membrane. The receptor tyrosine kinase family protein consists of an extra-cellular domain exposed to outside of cell, an intracellular domain exposed to cytoplasm of cell inside, and transmembrane domain positioned between the extra-cellular and intracellular domains and passing through cell membrane. A specific ligand binds to extra-cellular domain of the receptor. The intracellular domain of the receptor transfers an activating signal of the receptor activated by the ligand binding into the inside of cell. The receptor tyrosine kinase family protein has a tyrosine kinase activity domain in its c-terminal fragment exposed to outside of cell and when a specific ligand binds to its extra-cellular domain, the receptor tyrosine kinase protein is modified from monomer into dimer. Then, the tyrosine kinase activity is activated to phosphorylate tyrosines in their C-terminal on the dimer. Such tyrosine phosphorylation is the most important process to transfer the signal of an external stimulus inside cells. As such, the receptor in which the c-terminal tyrosines are phosphorylated generates a signal inside cells. The receptor having the tyrosine kinase activity transferring external stimulus inside cells has been widely known as such mechanism. Examples of the receptor

include EGFR, PDGFR, IR, IGFR, c-fms, VEGFR and the like.

DDR (Discoindin Domain Receptor) is one of receptor tyrosine kinase family having a tyrosine kinase activity. DDR is named in relation to the similarity to discoidin which is a protein attached onto lectin having the extra-cellular domain found in microbe. In case of animals including human beings, there are two types of DDR proteins, DDR1 type and DDR2 type proteins, which have similar amino acid sequences and encoded by different genes to each other. It was known in the early 1990's that Human being has DDR tyrosine kinase genes. However, researches for the function of the kinase have been attracted attention since the ligand binding thereto was found as collagen fiber for the first time.

In general, in many cases, diseases attacks in case where a genetic deformity or environmental effect excessively expresses a specific receptor tyrosine kinase, a specific receptor tyrosine kinase is kept activated due to a structural change regardless of an existence of the corresponding ligand, or the activity adjustment mechanism is damaged due to the over-generation of the corresponding ligand so as to greatly increase such activity or decrease it. Most typically, it has been found that the excessive activity of the receptor such as EGFR or PDGFR is one of critical factors of cancers. Also, it has been known that the increased activity of VEGFR deeply relates to cancer transfer and malignance. Recently, several drug companies developed EGFR specific inhibitory compound or VEGFR specific inhibitory compound and they are on clinical tests as various medical drugs for treating cancers, for example. Such EGFR and VEGFR specific inhibitors are all compounds inhibiting the kinase activity in the c-terminal of the receptor-type proteins. In particular, they are competitively attached onto a fragment of their kinase enzyme to which ATP is to

be attached. Accordingly, they interrupt the binding between the enzyme and the ATP to inhibit the enzymatic activity. It has been reported that abnormalities of the DDR2 kinase proteins relate to human intractable diseases, e.g., liver cirrhosis, rheumatism and so forth. The liver cirrhosis is a lesion caused due to accumulation of over-produced fiber collagens in liver. Recently, test results have revealed that the DDR2 expression is increased during the activation of the liver astrocyte in liver tissues, which is spotted as a critical factor causing the liver cirrhosis symptoms. This represents that the increase in the DDR2 expression plays an important role in the activation of the liver astrocyte and also is a main factor causing the liver cirrhosis. Also, it has been meaningfully known that the accumulation of the collagen proteins as the ligand of the DDR2 due to the liver cirrhosis and the continuous activation of the receptor-type DDR2 proteins due to the accumulation precipitate the liver cirrhosis.

The rheumatism can be said as another disease related to the collagen. The rheumatism is a disease that immunocytes around cartilages are continuously activated to increase an amount of cytokine (e.g., TNF-a) secretion, thereby to greatly increase the activity of MMP-1 as collagenase, resulting in destroying cartilaginous tissues. Cells in the cartilaginous tissues typically expressing MMP-1 proteins are synovial fibroblasts existing in the synovial membrane covering the cartilages. Generally, the proliferation and activation of such synovial fibroblast are well controlled in a normal condition. However, it has been recently reported that the increase in the activity of the DDR2 proteins causes an increased expression of the MMP-1 genes. The expression of the DDR2 proteins was observed in the synovial fibroblasts extracted from the cartilaginous tissues of a patient with rheumatism. It has also observed that if

such synovial fibroblasts are treated with several types of collagens, the promoter activity of the MMP-1 genes is increased. These can be considered as the increase in the DDR2 activity is one of direct causes for the rheumatic lesion.

Based upon the above facts, it can be inferred that a material specifically inhibiting the DDR2 tyrosine kinase activity can be used to treat liver cirrhosis or rheumatism.

On the other hand, it has been known that Src proteins are expressed from Src-genes identified as an oncogene of Rous sarcoma virus, a type of retrovirus. In general, the Src protein is known to perform adhesion, migration, proliferation and differentiation all related to cells.

The present invention has studied a tyrosine kinase activation factor of DDR2 proteins in a molecular level from facts that a main cause of liver cirrhosis, rheumatic lesion or arteriosclerotic lesion is an abnormal growth of fibroblasts at the affected region and the growth of the fibroblast family cells requires expression and activation of the DDR2 proteins. Accordingly, DDR2 protein kinase active domain is tyrosine-phosphorylated by the Src tyrosine kinase, thereby preparing a modified DDR2 kinase active domain protein. It has been found out that such tyrosine-phosphorylation increases the DDR2 tyrosine kinase activity, and thusly it has been proved that the tyrosine phosphorylation is functionally important for the DDR2 tyrosine kinase activity. Thus, such protein containing the tyrosine-phosphorylated kinase active domain prepared by the above method is very useful in discovering and developing medical drugs (compounds) for treating diseases caused by an excessive activity of DDR2 protein through developing a DDR2 kinase activity inhibiting compound, in that the DDR2 kinase activity is activated in the lesions, such as liver cirrhosis,

rheumatism, arteriosclerosis or the like.

[Technical object of the present invention]

As described above, the present invention aims to provide an effective target protein for developing medical drugs for treating diseases caused by an excessive activity of DDR2 tyrosine kinase, by demonstrating that the tyrosine phosphorylation functionally plays an important role in the DDR2 tyrosine kinase activity, by way of proving the increase in the DDR2 tyrosine kinase activity due to the tyrosine phosphorylation, in a tyrosine kinase active domain of the DDR2 protein, and a preparation method thereof.

[Construction of the present invention]

To achieve these and other advantages and in accordance with the purpose of the present invention, as embodied and broadly described herein, there is provided a use of DDR2 protein as a target material (protein) for developing a medical drug for treating diseases caused by tyrosine-phosphorylated DDR2 protein, the DDR2 protein having an increased kinase activity due to the tyrosine phosphorylation induced in the tyrosine kinase active domain using tyrosine kinase activity of Src protein, and a method for preparing the DDR2 protein having the tyrosine phosphorylation induced in the tyrosine kinase active domain using the tyrosine kinase activity of the Src protein.

There is no specific limitation on the method for phosphorylating the tyrosine in the tyrosine kinase active domain of the DDR2 protein using the Src protein. For example, such tyrosine phosphorylation can be achieved by mixing a purified Src protein with the DDR2 protein under a typical condition, or co-expressing the DDR2 kinase domain protein with the Src tyrosine kinase

protein in host cells such as insect cells.

In one embodiment of the present invention, the method for phosphorylating the tyrosine at the DDR2 kinase active domain may include the following steps of:

amplifying cDNA which encodes an amino acid sequence sufficiently covering the DDR2 kinase domain protein, randomly performing a typical affinity tagging using, for example, glutathione-S-transferase (GST) gene, in order to facilitate the purification of the expressed DDR2 protein, fusing such tagged gene with the DDR2 gene, introducing the fused genes into an appropriate virus, and generating a recombinant virus encoding the DDR2 protein;

amplifying the cDNA which encodes the amino acid sequence sufficiently covering Src protein, introducing the amplified cDNA into an appropriate virus, and generating a recombinant virus encoding Src tyrosine kinase;

simultaneously infecting the obtained DDR2 protein encoding recombinant virus and Src protein encoding recombinant virus into a host cell at an appropriate rate, co-expressing the proteins, and expressing the DDR2 protein having the tyrosine activation induced due to the tyrosine kinase activity of the co-expressed Src protein; and

purifying the DDR2 protein having the tyrosine phosphorylation induced.

Also, the present invention provides a DDR2 protein with a tyrosine kinase active domain having a modified activity due to the tyrosine phosphorylation according to the above method, and the use of the DDR2 protein in the studies on developing medical drugs for treating a disease through a screening for activity inhabiting compounds or a protein structure analysis of the DDR2 tyrosine kinase active domain, upon developing medical drugs caused by the

tyrosine phosphorylation in the tyrosine kinase active domain of the DDR2 protein. The tyrosine-phosphorylated DDR2 protein according to the tyrosine phosphorylation method of the DDR2 kinase of the present invention can be used as a more effective material in developing medical drugs for treating diseases caused by the modification of the tyrosine kinase activity due to the tyrosine phosphorylation in the tyrosine kinase active domain of the DDR2 protein, particularly, diseases mainly caused by a fibroblast family cell growth due to an excessive activation of the DDR2 tyrosine kinase.

A tyrosine phosphorylation method in a tyrosine kinase active domain of a DDR2 protein according to the present invention will now be described in detail in accordance with a preferred embodiment.

DDR2 protein is a kind of a receptor protein attached to a plasma membrane. As mentioned above, the DDR2 protein consists of three domains, i.e., an extracellular domain (i.e., N-terminal region), a transmembrane domain and an intracellular domain (i.e., C-terminal region) exposed to cytosol. For example, a human DDR2 protein (SEQ ID NO: 1) consists of the extracellular domain generally exposed to the outside of cell, mainly consisting of amino acids from position 1 to position 399, the transmembrane domain consisting of 22 amino acids (ILIGCLVAIIFILLAIIVIILW; SEQ ID NO: 2) following to position 399, and the intracellular cytosolic domain (C-terminal region) comprising the tyrosine kinase domain consisting of amino acids from position 441 to position 855 (SEQ ID NO: 3). In the present invention, in order to study the change in the tyrosine kinase activity of the DDR2 protein by the Src tyrosine kinase, among a cDNA for human DDR2 protein, a cDNA fragment which encodes the amino acids from position 441 to position 855 (the end of the C-terminal) sufficiently covering the

tyrosine kinase active domain of the DDR2 protein may be amplified by PCR, and introduced into an appropriate virus by a conventional method (e.g., the cDNA fragment amplified is cloned in an appropriate expression vector and then the obtained expression vector is introduced into an appropriate virus), so as to generate a recombinant virus expressing the DDR2 protein.

Preferably, in order to facilitate the purification of the expressed DDR2 protein, the cDNA fragment comprising the sufficient DDR2 tyrosine kinase active domain protein is fused with an appropriate gene for an affinity tagging, and the fused DNA fragment is introduced into a virus. Viruses used in the affinity tagging may include glutathione-S-transferase (GST) gene, thioredoxin gene, histidine oligomer and the like. Preferably, baculovirus having a strong promoter and its expression vector may be used as the virus to be recombined and the expression vector.

In the preferable embodiment of the present invention, the cDNA fragment containing the DDR2 tyrosine kinase active domain protein is attached to the C-terminal coding region (3 ' region) of the glutathione-S-transferase gene, and the fused DNA fragment is cloned into a baculovirus expression vector pBacPAK8 (Clontech, USA) by using a conventional gene recombination technique, to construct a recombinant baculovirus capable of producing a fused protein of the glutathione-S-transferase and the DDR2 tyrosine kinase domain protein (CLONTECH BacPAK™ Baculovirus Expression System User Manual, PT1260-1(PR95847), Published 12 May 1999. Catalog # K1601-1).

The DDR2 protein to be used is not limited to human DDR2 protein, and the cDNA may not be limited in the number of amino acids encoded thereby within a range containing the DDR2 tyrosine kinase coding region. Also, since

the fusing of the DDR2 gene fragment with the glutathione-S-transferase (GST) gene is only for facilitating the purification of the expressed DDR2 protein, such fusing process is not always essential for the present invention. The DDR2 protein may be used in a fused form according to its utility after the purification, or it may be used after removing the GST portion by using an appropriate protease.

In the present invention, in order to induce the tyrosine phosphorylation at the DDR2 tyrosine kinase active domain, a gene (SEQ ID NO: 4) encoding a full-length Src tyrosine kinase is amplified by PCR, and cloned into an appropriate expression vector. The cloned gene is introduced into an appropriate virus, to generate a recombinant virus encoding the Src tyrosine kinase. As mentioned above, the baculovirus may be used as the virus to be recombined.

Upon co-infecting the recombinant virus which encodes the obtained DDR2 kinase active domain or the fused protein of the DDR2 kinase active domain with the glutathione-S-transferase and the recombinant virus encoding the Src kinase in an appropriate host cell at a proper rate and co-expressing them, the tyrosine of the DDR2 kinase active domain is phosphorylated by the tyrosine phosphorylation activity of the expressed Src protein, so as to obtain the tyrosine phosphorylation-induced DDR2 kinase. In the preferred embodiment of the present invention, when generating the recombinant virus for which the baculovirus is used, insect cell sf9 (Clontech, USA) which is conventionally used as a host cell for protein expression by the baculovirus may be used. Also, for the simultaneous infection into the recombinant virus encoding the DDR2 kinase active domain or the fused protein of the DDR2 kinase active domain and the glutathione-S-transferase and the recombinant virus encoding the Src kinase, if

the rate of the Src kinase encoding recombinant virus is increased, the amount of the expressed Src protein is increased to enhance the phosphorylation efficiency. On the other hand, the rate of the DDR2 kinase active domain encoding virus is decreased, resulting in the decrease in the amount of the expressed DDR2 protein, thereby reducing the amount of recollected DDR2 protein. Considering such point, the combination ratio of the recombinant virus encoding the DDR2 kinase active domain or the fused protein of the DDR2 kinase active domain with the glutathione-S-transferase and the Src kinase encoding recombinant virus is based on the number of the viruses in the range of 19:1 or 1:19 (the number of recombinant viruses encoding the DDR2 kinase active domain (or the fused protein of the DDR2 kinase active domain with the glutathione-S-transferase) : the number of recombinant viruses encoding the Src kinase).

The obtained tyrosine phosphorylation-induced DDR2 kinase fused with the glutathione-S-transferase may be purified in a high purity by an affinity chromatography according to a conventional method using glutathione-attached beads.

In the preferred embodiment of the present invention, the expression of the fused protein of the glutathione-S-transferase and the DDR2 kinase active domain, and the Src tyrosine kinase, in insect cells, may be achieved by simultaneously infecting two recombinant baculoviruses encoding each of them into insect cell sf9, at the proper combination ratio and the MOI (Multiplicity of infection) of 1 to 10, and then, maintaining for 24 to 72 hours. As mentioned above, the Src protein and the GST-DDR2 fused protein are co-expressed, followed by lysing the cell, and a glutathione agarose affinity column chromatography is carried out according to a conventional method, thereby

purifying the DDR2 tyrosine kinase domain fused with the glutathione-S-transferase.

Fig. 1 shows a result obtained by purifying the fused protein of glutathione-S-transferase and DDR2 tyrosine kinase active domain through a glutathione agarose bead column chromatography, performing an electrophoresis in 10 % polyacrylamaide gel, and then staining with coomassie. As shown in Fig. 1, the purified protein has the molecular weight of 75,000 Da, which is an expected molecular weight.

For a control experiment in order to check the effect due to the co-expression of the DDR2 tyrosine kinase active domain and the Src, according to the same method as used above, the fused protein of the glutathione-S-transferase and the DDR2 tyrosine kinase active domain is only expressed. It has been checked, through the western blotting test using a Src protein specific antibody, the Src protein encoding recombinant baculovirus used in this experiment expresses the Src protein, and the result has been also shown in Fig. 2. In the embodiment of the present invention, the human c-Src gene may be used; however, the same results can be obtained by using Src gene of other species, a modified v-Src gene or the like.

As an interesting result in the present invention, as shown in Fig. 3a, it has been checked, through the western blotting test using a phosphorylated tyrosine specific antibody, that when the fused protein of the glutathione-S-transferase (GST) and the tyrosine kinase active domain is co-expressed together with the Src tyrosine kinase, the tyrosine of the fused protein is phosphorylated and modified. Fig. 3a shows that, by the western blotting method using the phosphorylated tyrosine (p-tyrosine) specific antibody, when the fused protein of

the glutathione-S-transferase and the DDR2 tyrosine kinase active domain is expressed in insect cell, the induction of the tyrosine phosphorylation of the DDR2 tyrosine kinase active domain depends on whether the fused protein is co-expressed together with the Src tyrosine kinase.

Here, unlike the fused protein of the glutathione-S-transferase and the DDR2 tyrosine kinase active domain purified after the co-expressed with the Src protein, the tyrosine phosphorylation is never induced in the fused proteins of the GST with DDR1, CDK4 and CDK1 used as control groups when they are co-expressed with the Src tyrosine kinase, and the results can be seen in Fig. 3b. As shown in Fig. 3b, it can be proved that the tyrosine phosphorylation induced by the co-expression with the Src tyrosine kinase is specific to the DDR2 tyrosine kinase active domain.

In the above testing method, when co-infecting the DDR2 protein encoding baculovirus and the Src protein encoding baculovirus into the host cell with the ratio therebetween being varied, it can be seen that as the ratio of the Src expression virus is higher, the higher level of the tyrosine phosphorylation at a unit amount of the DDR2 tyrosine kinase domain is obtained. This means that the induction of the tyrosine phosphorylation at the DDR2 kinase domain by the Src protein depends on the concentration of the Src protein. Fig. 4 shows the result obtained by performing the co-infection of the DDR2 kinase encoding virus and the Src protein encoding virus at the changeable ratio therebetween; quantitatively purifying the expressed DDR2 kinase active domain; and performing the western blotting test using the phosphorylated tyrosine specific antibody. As shown in Fig. 4, the tyrosine phosphorylation at the DDR2 tyrosine kinase domain was meaningfully induced, when the DDR2 kinase encoding virus

and the Src protein encoding virus are co-expressed at the ratio of 19:1.

In order to show the functional difference between the DDR2 tyrosine kinase protein in which tyrosine phosphorylation is induced by the co-expression with the Src tyrosine kinase and the DDR2 tyrosine kinase protein in which no tyrosine phosphorylation is induced, the enzymatic activities of both the tyrosine-phosphorylated DDR2 tyrosine kinase and the non-phosphorylated DDR2 tyrosine kinase have been measured and compared respectively, and the results are shown in Fig. 5. Fig. 5 shows the results obtained by measuring the kinase enzymatic activity of the DDR2 tyrosine kinase active domain protein in which the tyrosine phosphorylation is induced by the co-expression with the Src tyrosine kinase and the kinase enzymatic activity of the DDR2 tyrosine kinase active domain protein in which no tyrosine phosphorylation is induced due to its sole expression followed by the purification. According to the results by measuring and comparing the tyrosine enzymatic activities by typical methods (Refer to Promega, 2001, Catalog #15.18) using a biotin-attached poly(D4Y) substrate (Promega, U.S.A) which is conventionally used in a method for measuring the tyrosine kinase enzymatic activity, as shown in Fig. 5, the DDR2 tyrosine kinase in which tyrosine has been phosphorylated has increased kinase activity by about 3 to 10 times comparing to the control protein in which no tyrosine phosphorylation has been induced. In measuring auto-phosphorylation activity using the DDR2 kinase active domain, it has been shown that the auto-phosphorylation activity of the tyrosine-phosphorylated DDR2 kinase is increased by about 3 to 10 times as well.

As one measurement for searching the molecular reason of such increase in the kinase activity, the change in Km value for ATP as one of substrates for

the kinase enzyme was measured. To this end, Fig. 6 shows the results obtained by determining the variation of a reaction depending on the concentration of ATP, conducting a reciprocal plotting (Lubert Stryer, Biochemistry Fourth Edition, Published in Seoul Foreign Books, pp. 202-205), which is used for measuring the Km value for the typical substrate for the enzyme, and checking the variation of a reciprocal (Km value) of x-axis intercept. As shown in Fig. 6, with comparison to that of the non-phosphorylated control protein, Km value of the tyrosine-phosphorylated DDR2 kinase protein due to the Src protein has been decreased. Therefore, it can be checked that, with comparison to that of the non-phosphorylated DDR2 kinase active domain protein, the increased enzymatic activity of the tyrosine-phosphorylated DDR2 kinase domain protein, results from the increased binding capacity to the substrate ATP.

As mentioned above, in developing medical drugs by discovering an inhibitor against the DDR2 protein, in case of targeting a compound typically having an inhibitory mechanism by ATP competitive mechanism, it can be seen that the tyrosine-phosphorylated DDR2 protein is a new target molecularly different from the non-phosphorylated control DDR2 protein. This can be considered as a typical case of research for developing a modified new target protein through the study on a property variation depending on the variation after the expression of the target protein in the recent trend of research for developing new medical drugs through a proteome study.

After the expression from mRNA, a protein is chemically modified in several forms. Such forms of the chemical modification have been known, for example, including sugar group substitution, phosphorylation, cleavage by protease and the like. Such forms of modification depend on environments in

which cells are placed. Also, different forms of chemical modification may show great difference in the protein activities. A representative example among several chemical modifications after the protein expression may be the phosphorylation. In particular, the activity of the protein kinase playing an important role in a cellular signal transmission system may be greatly affected by the phosphorylation.

Many proteins are modified in their activities under a diseased state, and such activity-modified proteins are considered as targets for developing medical drugs to treat diseases. The modification of the protein to be used as a target may cause the change in a binding way of a low molecular compound attached to the protein. Such reason may be related to that even if proteins are produced from the same gene, they can be different target proteins according to their chemically modified patterns after their expression, which is the important conclusion of the recent proteomic study. Considering this, it can be said that the tyrosine-phosphorylated DDR2 protein can be useful as a target for developing medical drugs to treat diseases, which is different from the non-phosphorylated DDR2 protein.

Also, the experimental facts that, in the DDR2 kinase, the ATP-binding site, which is generally considered as a main target point in developing a kinase inhibitor, may be structurally changed due to the affect of the Src tyrosine kinase suggest that, the tyrosine-phosphorylated DDR2 kinase protein provided in the present invention can be a new target protein for a disease caused by the abnormal DDR2 kinase activity.

The present invention will now be described in more detail with reference to preferred embodiments as follows. However, the present invention may not

limited by those preferred embodiments.

Embodiment 1

Expression and purification of DDR2 tyrosine kinase active domain and Src protein in insect cells

The C-terminal region encoding human DDR2 gene having amino acids from 441 to 855 was PCR-amplified using 5′ primer of ccc gga tcc atg aca gtc agc ctt tcc ct (SEQ ID NO: 7) and 3′ primer of ggg tct aga tca ctc gtc gcc ttg ttg aag (SEQ ID NO: 7), by using human DDR2 full-length DNA as a template, thus to be cut into Bam HI and Xba I cutting sites. The obtained DDR2 DNA fragment was in frame fused between Bam H1 and Xba I sites at 3′ position of the GST gene of the pBacPAK8-GST fused vector, which was generated by recombining multiple cloning site of GST gene region and 3′ region thereof, the GST from pGEX4T-1 vector (cat #: 27-4580-01) purchased from Amersham Biosciences (U.S.A), with Bam HI site of an insect cell expression vector pBacPak8 (Clontech, USA), thereby generating pBacPAK-GST-DDR2 KD (kinase domain) vector.

The recombinant baculovirus using the expression vector was constructed using baculovirus generation kit (Catalog # K1601-1, Clontech, USA) by a typical method (CLONTECH BacPAKTM Baculovirus Expression System User Manual, PT1260-1 (PR95847), Published 12 May, 1999).

Such obtained recombinant virus was cultured for about 24 to 72 hours after the infection into sf9 insect cell (Clontech, U.S.A.) with the MOI of 10. Cells were then lysed to purify an expressed protein. The cultured cells were harvested, and the harvested cells were suspended in a solution consisting of 20mM Tris-HC (pH 7.5), 100mM NaCl, 50mM NaF, 1 m orthovanadate, 1 mM DTT and

protease inhibitor cocktail (BioRad, U.S.A) and then sonicated to thereafter lyse the cells. After the centrifugation at 12,000 g for 30 minutes, the lysate was applied to the glutathione agarose bead affinity column filled with buffer solution consisting of 20mM Tris-HC (pH 7.5), 100mM NaCl, 50mM NaF and 1mM DTT, and proteins which were not sufficiently attached to the column were washed using the column buffer solution. Afterwards, the attached proteins were eluted using a solution consisting of 20mM glutathione and the obtained proteins were concentrated using a centrifugal filter. The eluted proteins were run in 10% polyacrylamaide gel electrophoresis (PAGE). The results were shown in Fig. 1. As shown in Fig. 1, the purified protein has the molecular weight of 75,000 Da, which is an expected molecular weight corresponding to the DDR2 tyrosine kinase domain fused with the glutathione-S-transferase.

Also, all human Src cNDA gene was PCR-amplified from the full length wild-type human Src cDNA expression vector (Src cDNA in pUSEamp: Catalog #21-114) as a template, using 5′ primer of GGGGGATTCGACG-GATCGGGAGATCTCCCG (SEQ ID NO: 7) and 3′ primer of CCCGAATTCGAC GTC AGG TGG CAC TTT TCG GGG (SEQ ID NO: 8). the amplified gene is simultaneously cut into Bam H1 and Ecok I, and was cloned into Bam HI and EcoRI sites existing in the multiple cloning site of the pBacPak8 vector (Clontech, USA) as insect cell expression vector. As described above, by using the baculovirus generation kit (Clontech, USA), the recombinant baculovirus encoding the Src tyrosine kinase using the Src tyrosine kinase expression vector was generated.

It has been checked that such generated recombinant baculovirus has expressed the human Src gene in insect cells through the western blotting using

human Src specific antibody (Santa cruz, USA), and the result was shown in Fig. 2.

Embodiment 2

Induction of tyrosine phosphorylation of DDR2 kinase active domain protein through co-expression of Src protein and DDR2 kinase active domain and confirmation thereof

Two recombinant baculovirus respectively having the Src protein encoding gene and the DDR2 kinase active domain encoding gene were infected into sf9 insect cell, by setting the MOU of the all viruses to 1:10 at a ratio of 1:1 or with varying such combination ratio (e.g., the DDR2 protein encoding virus : Src protein encoding virus = 1:1 to 19:1). The viruses were cultured for 24 to 72 hours to express proteins. The cells were then lysed, and the DDR2 kinase active domain protein was purified using the glutathione agarose bead column chromatography as same in the embodiment 1. The purified protein was subjected to an electrophoresis in 10 % polyacrylamaide gel.

After completing the electrophoresis, the protein developed in the gel was transferred to nitrocellulose, and then subjected to a western blotting using a phosphorylated tyrosine specific antibody (santa cruz, USA), to measure the chemical luminescence signal using x-ray film. For the control, the chemical luminescence signal was measured by the method as mentioned above even in the case where only the fused protein of the glutathione-S-transferase and the DDR2 protein was solely expressed, and the results was shown in Figs. 3 and 4.

Embodiment 3

Measurement for DDR2 tyrosine kinase activity and Km value for ATP

The reaction was performed for 30 minutes using 100 ng of DDR2 tyrosine kinase in 20 μl of reaction mixture containing Tris-HCl (pH 7.5), 5 mM $MgCl_2$, a biotin-attached poly(D4Y) substrate (Promega, U.S.A), 10 mM ATP and 0.2 uCi of P 32-gamma-ATP. The reaction was stopped by adding a half volume of 30% phosphate solution. The stopped mixture was spotted on avidine coated membrane (Promega, USA), and then washed with 10 mM Tris-HCl (pH 8.0) and 100 mM NaCl five times. The radioactivity generated from the phosphorylated peptide attached to the membrane was visualized using BAS radioactivity image analyzer (Kodak), and the enzymatic reaction was quantitated.

For measuring the autophosphorylation activity, under the same conditions, the reaction was performed in a reaction mixture excluding 2 μl of poly(D4Y)n. The reaction mixture was subjected to the electrophoresis in 10 % PAGE gel and stained using coomassie, to confirm the existence of the DDR2 kinase active domain protein. The gel was then dried. The autophosphorylation of the DDR2 kinase active domain was measured by autoradiography using x-ray film.

According to the present invention, the above mentioned experiments were performed with respect to the tyrosine-phosphorylated DDR2 kinase and non-phosphorylated control DDR2 protein (i.e., DDR2 protein purified after the sole expression of GST-DDR2 fused protein), and the results was shown in Fig. 5. As shown in Fig. 5, with comparison to that of the non-phosphorylated control protein, the phosphorylation activity of the tyrosine-phosphorylated DDR2 protein was increased by about 3 to 10 times.

In order to determine Km value for ATP of the DDR2 kinase active domain

protein, through the enzyme reaction velocity measurement based on the above methods, the variations of the reaction velocities for the tyrosine-phosphorylated DDR2 protein according to the present invention and the non-phosphorylated control DDR2 protein were measured. Km value was determined by the reciprocal of x-axis intercept of each ATP concentration on a linear graph obtained after spotting a reciprocal of a reaction velocity for a reciprocal of each ATP concentration, and the result was shown in Fig. 6. As shown in Fig. 6, Km value for the ATP of the tyrosine-phosphorylated DDR2 protein due to the Src was decreased with comparison to that of the non-phosphorylated control protein.

[Effect of the invention]

The present invention provides a method for preparing a protein having an increased DDR2 kinase activity and a DDR2 kinase active domain protein having an increased kinase activity, whereby such protein can be useful as a target protein for discovering a medical drug in developing new drugs for treating the diseases mainly caused by the growth of a fibrotic cells, such as liver cirrhosis, arteriosclerosis, rheumatism and the like.

What is claimed is:

1. A target material for developing a medical drug for treating a disease caused by DDR2 protein having a tyrosine kinase activity modified due to tyrosine phosphorylation, the target material including DDR2 protein having DDR2 tyrosine kinase active domain having the tyrosine phosphorylation induced due to a tyrosine kinase activity of Src protein.

2. The material of claim 1, wherein the tyrosine phosphorylation at the DDR2 tyrosine kinase active domain due to the Src protein is induced by co-expression, in a host cell, of a recombinant virus in which an amino acid sequence encoding gene sufficiently covering the DDR2 kinase active domain is introduced and a recombinant virus in which Src protein encoding gene is introduced.

3. The material of claim 1 or 2, wherein the disease includes liver cirrhosis, arteriosclerosis or rheumatism.

4. A method for preparing DDR2 protein having a tyrosine phosphorylation induced kinase active domain, the method comprising:

amplifying cDNA encoding an amino acid sequence sufficiently covering the kinase active domain of the DDR2 protein, introducing the amplified cDNA into a virus, and generating a recombinant virus encoding the DDR2 protein;

amplifying cDNA encoding an amino acid sequence sufficiently covering Src protein, introducing the amplified cDNA into a virus, and generating a

[Drawings]

Fig. 1

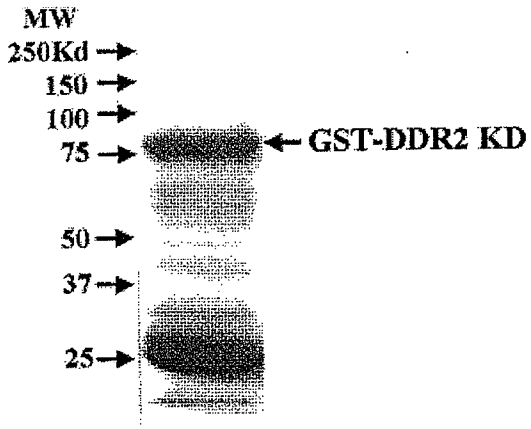


Fig. 2

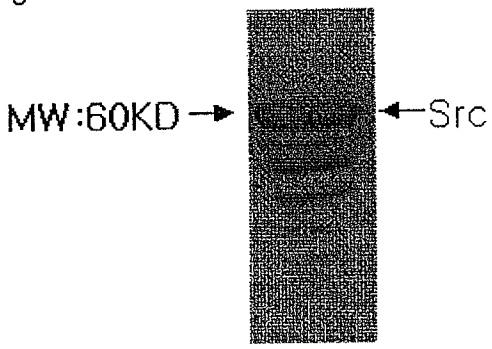


Fig. 3a

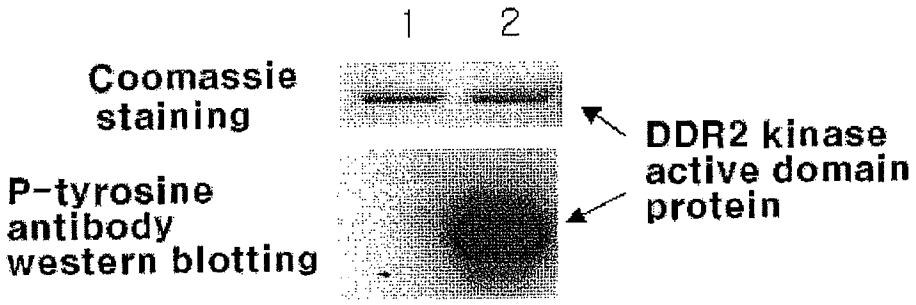


Fig. 3b

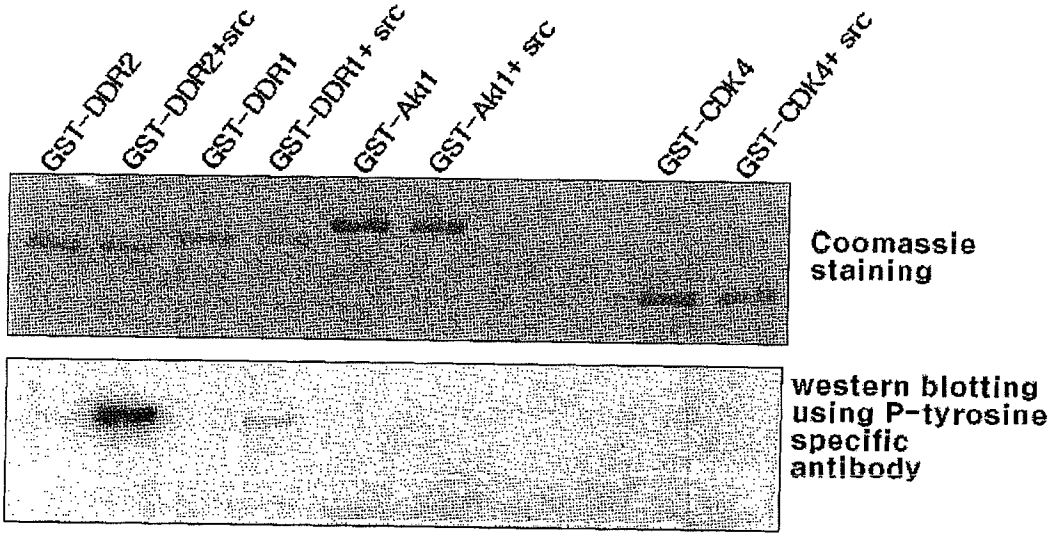


Fig. 4

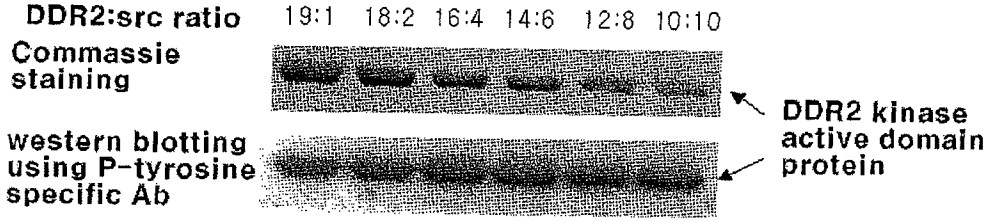


Fig. 5

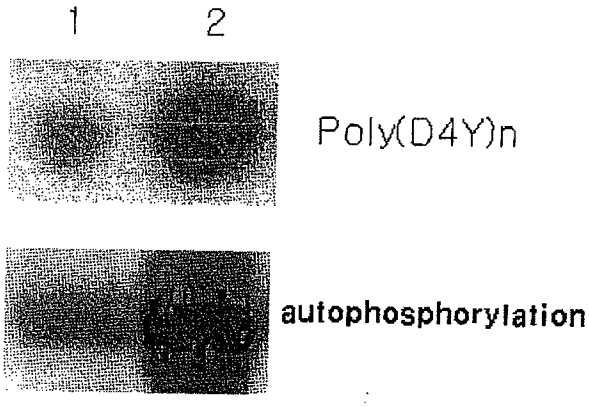
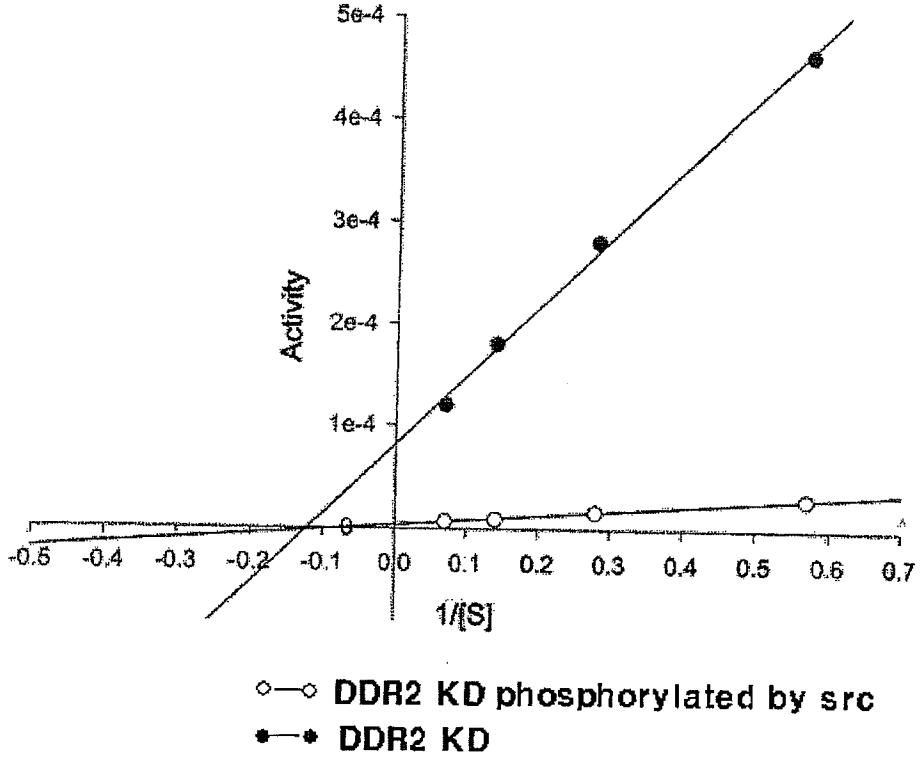


Fig. 6



[Sequence List]

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Ser Glu Glu Gly Asp Gly Ala Trp Cys Pro Glu Ile Pro Val Glu Pro
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Asp Asp Leu Lys Glu Phe Leu Gln Ile Asp Leu His Thr Leu His Phe
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Glu Phe Ala Pro Met Tyr Lys Ile Asn Tyr Ser Arg Asp Gly Thr Arg

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Trp Ile Ser Trp Arg Asn Arg His Gly Lys Gln Val Leu Asp Gly Asn
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Ala Arg Phe Val Arg Phe Ile Pro Val Thr Asp His Ser Met Asn Val
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Cys Met Arg Val Glu Leu Tyr Gly Cys Val Trp Leu Asp Gly Leu Val
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Ser Tyr Asn Ala Pro Ala Gly Gln Gln Phe Val Leu Pro Gly Gly Ser
195 200 205

Ile Ile Tyr Leu Asn Asp Ser Val Tyr Asp Gly Ala Val Gly Tyr Ser
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Met Thr Glu Gly Leu Gly Gln Leu Thr Asp Gly Val Ser Gly Leu Asp
225 230 235 240

Asp Phe Thr Gln Thr His Glu Tyr His Val Trp Pro Gly Tyr Asp Tyr
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Val Gly Trp Arg Asn Glu Ser Ala Thr Asn Gly Tyr Ile Glu Ile Met
260 265 270

Phe Glu Phe Asp Arg Ile Arg Asn Phe Thr Thr Met Lys Val His Cys

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Asn Asn Met Phe Ala Lys Gly Val Lys Ile Phe Lys Glu Val Gln Cys
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Tyr Phe Arg Ser Glu Ala Ser Glu Trp Glu Pro Asn Ala Ile Ser Phe
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Pro Leu Val Leu Asp Asp Val Asn Pro Ser Ala Arg Phe Val Thr Val
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355 360 365

Ala Met Tyr Asn Asn Ser Glu Ala Leu Pro Thr Ser Pro Met Ala Pro
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Thr Thr Tyr Asp Pro Met Leu Lys Val Asp Asp Ser Asn Thr Arg Ile
385 390 395 400

Leu Ile Gly Cys Leu Val Ala Ile Ile Phe Ile Leu Leu Ala Ile Ile
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Val Ile Ile Leu Trp Arg Gln Phe Trp Gln Lys Met Leu Glu Lys Ala
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Arg Lys Leu Pro Glu Phe Ala Pro Gly Glu Glu Glu Ser Gly Cys Ser

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Tyr Ala Glu Ala Asp Ile Val Asn Leu Gln Gly Val Thr Gly Gly Asn
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Thr Tyr Ser Val Pro Ala Val Thr Met Asp Leu Leu Ser Gly Lys Asp
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Leu Gly Glu Gly Gln Phe Gly Glu Val His Leu Cys Glu Val Glu Gly
130 135 140

Met Glu Lys Phe Lys Asp Lys Asp Phe Ala Leu Asp Val Ser Ala Asn
145 150 155 160

Gln Pro Val Leu Val Ala Val Lys Met Leu Arg Ala Asp Ala Asn Lys
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Asn Ala Arg Asn Asp Phe Leu Lys Glu Ile Lys Ile Met Ser Arg Leu
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Lys Asp Pro Asn Ile Ile His Leu Leu Ser Val Cys Ile Thr Asp Asp
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Pro Leu Cys Met Ile Thr Glu Tyr Met Glu Asn Gly Asp Leu Asn Gln
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Phe Leu Ser Arg His Glu Pro Pro Asn Ser Ser Ser Ser Asp Val Arg
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Thr Val Ser Tyr Thr Asn Leu Lys Phe Met Ala Thr Gln Ile Ala Ser
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Gly Met Lys Tyr Leu Ser Ser Leu Asn Phe Val His Arg Asp Leu Ala
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Phe Gly Met Ser Arg Asn Leu Tyr Ser Gly Asp Tyr Tyr Arg Ile Gln
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Leu Gly Lys Phe Thr Thr Ala Ser Asp Val Trp Ala Phe Gly Val Thr
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Gly Arg Gln Thr Tyr Leu Pro Gln Pro Ala Ile Cys Pro Asp Ser Val
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aagctcttcg gaggcttcaa ctcctcggac accgtcacct ccccgcagag ggcgggggct 240

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